Reference: *Biol. Bull.* **188**: 1–4. (February/March, 1995)

Responses of the Medaka Fish Egg (*Oryzias latipes*) to the Photolysis of Microinjected Nitrophenyl-EGTA, a Photolabile Calcium Chelator

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Photolabile calcium chelators (calcium cages) can be used to elevate cytosolic [Ca^{2+}] at specific sites and times (1, 2, 3). They have been especially valuable in flash photolysis studies of muscle contraction (2) and secretion (4, 5). In the present report, I describe several responses of medaka eggs to the photolysis of microinjected nitrophenyl-EGTA (NP-EGTA), a new calcium cage (6). When unfertilized eggs injected with NP-EGTA were irradiated with ultraviolet irradiation in a small region of the egg, the eggs were activated and ooplasm within the irradiated region contracted and accumulated there. Eggs into which NP-EGTA was injected could also be fertilized. Subsequent irradiation of such eggs, in addition to causing the contraction and accumulation of ooplasm, also caused a global contraction of dividing blastomeres and the contraction and blebbing of embryonic cells for up to 4 days after fertilization. Injection of NP-EGTA had no apparent effect on the maturation of fertilized eggs, which developed normally and hatched.

The methods for dissection of gonads from breeding medaka, preparation of gametes, and *in vitro* fertilization of eggs have been described previously (7). Gonads, gametes, and zygotes were prepared in a balanced saline solution (BSS: 111 m*M* NaCl; 5.37 m*M* KCl; 1.0 m*M* $CaCl_2$; 0.6 m*M* $MgSO_4$; 5 m*M* HEPES, pH 7.3). A normalized time (T_n) scale in which the time between fertilization and the beginning of cytokinesis is 1 unit was used to indicate the relative temporal positions of events. A total of 80 eggs from 6 females were used in these experiments; 44 of the eggs were monitored in detail and an additional 36 were monitored intermittently. The experiments were conducted at room temperature (23°–25°C).

Received 30 August 1994; accepted 14 October 1994.

Methods described previously (8, 9, 10) were used to microinject 1.4–5.6 nl of an aqueous solution of NP-EGTA/Ca^{2+} (50 m*M* NP-EGTA, tetrapotassium salt; 39.6 m*M* $CaCl_2$; 10 m*M* HEPES, pH 7.3) into the thin peripheral layer of ooplasm. Assuming an accessible volume of 27.6 nl (9), injection of these volumes would give a final ooplasmic concentration of 2.5–10.0 m*M* NP-EGTA. After microinjection, the eggs were either placed in a darkened cabinet for subsequent use or fertilized within 5 min. During the experiments, the laboratory was only dimly illuminated with incandescent lamps.

For microscopic observation and irradiation, the eggs were transferred to a microscope slide on which a cover glass was supported by four pillars of petroleum jelly (7). An Osram 100 W mercury arc lamp was used to irradiate the eggs with ultraviolet light. The light from the lamp was passed through a filter cube (Omega Optical) containing a 360 DF 40 exciter filter, a DC 405 dichroic mirror, and a 486 DF32 barrier filter. An octagonal diaphragm was used to control the size of the light beam (in most experiments, it was either 200 μm or 475 μm), and neutral density filters (Omega Optical) were used to reduce the light intensity by either 34-fold or 286-fold. Ultraviolet light was projected onto the egg via one of three objective lenses (Nikon): Plan 4×, N.A. = 0.1; Fluor/Ph 2 DL 10×, N.A. = 0.5; Fluor/Ph 3 DL 20×, N.A. = 0.75. In most experiments, the equatorial region of the egg—that is, a region along a meridian and midway between the animal and vegetal poles—was illuminated *en profil* (an edge of the egg was irradiated) via the 10× objective lens. Light intensity was measured with a UVX radiometer with a long wave sensor (UVP, Inc.). Given a light intensity of 523 μW cm^{-2} (referred to as "high intensity" hereafter) and assuming that all the ultraviolet light was of wavelength 360 nm, I calculated an incident light intensity of

4.9×10^9 quanta second^{-1} μm^{-2} (10× objective lens, no neutral density filter). To monitor the eggs during irradiation, they were transilluminated with light from a quartz-halogen lamp, using a heat filter (KG5) and a 486 DF32 filter, and the images were recorded via a SIT camera (Dage/MTI) and a time-lapse videocassette recorder.

To monitor the development of fertilized eggs after the first cell cycle, they were transferred to embryo rearing medium (17 m*M* NaCl; 0.4 m*M* KCl; 0.3 m*M* $CaCl_2$; 0.67 m*M* $MgSO_4$; 0.001 g/l methylene blue).

When unfertilized eggs into which 1.4–5.6 nl NP-EGTA had been injected were irradiated with UV light, they activated within 16 ± 3 s ($\bar{X}$ ± SD, n = 5), as evidenced by the exocytosis of cortical vesicles. Exocytosis began within the irradiated region and spread as a wave over the rest of the egg. Eggs were photoactivated even after the light intensity was reduced 34-fold with a neutral density filter; but when a second neutral density filter was added, reducing the light intensity an additional 8.5-fold, the eggs were not activated even after 3 min of continuous irradiation. Irradiation of unfertilized eggs that had not received NP-EGTA did not cause them to activate.

Continued irradiation of photoactivated eggs caused both ooplasm and oil droplets to accumulate in and next to the irradiated zone (Fig. 1A). Staining with rhodamine phalloidin showed that these accumulations of ooplasm contained filamentous actin (F-actin, Fig. 1B). Such accumulations of ooplasm and F-actin were identified in 18 eggs, 13 of which had been photoactivated and 5 of which had been fertilized. Moreover, the caps of ooplasm formed in eggs into which either 1.4 nl or 4.2 nl of NP-EGTA had been injected and in eggs that were irradiated either intermittently (5 s on/115 s off) with a high intensity of light or continuously with a 34-fold lower light intensity. However, when the light intensity was lowered 289-fold, ooplasm neither contracted nor accumulated in the irradiated zone. When UV irradiation was intermittent, the ooplasm within the irradiated zone usually contracted each time it was irradiated. For example, the region in the egg shown in Figure 1 was irradiated 29 times for 5 s and contracted 16 times, and a sibling egg contracted each of the 31 times it was irradiated for 5 s. Each contraction appeared to pull ooplasm and nearby oil droplets toward the irradiated region. Eggs that were parthenogenetically activated by the injection itself and grown in the dark segregated normally (as do eggs that have been parthenogenetically activated by pricking; Fluck, unpub. obs.), with a cap of ooplasm forming at the animal pole and the oil droplets segregating toward the vegetal pole.

Eggs into which NP-EGTA had been injected could also be fertilized. In most fertilized eggs, cortical vesicles in one small region of the egg, presumably near the in-

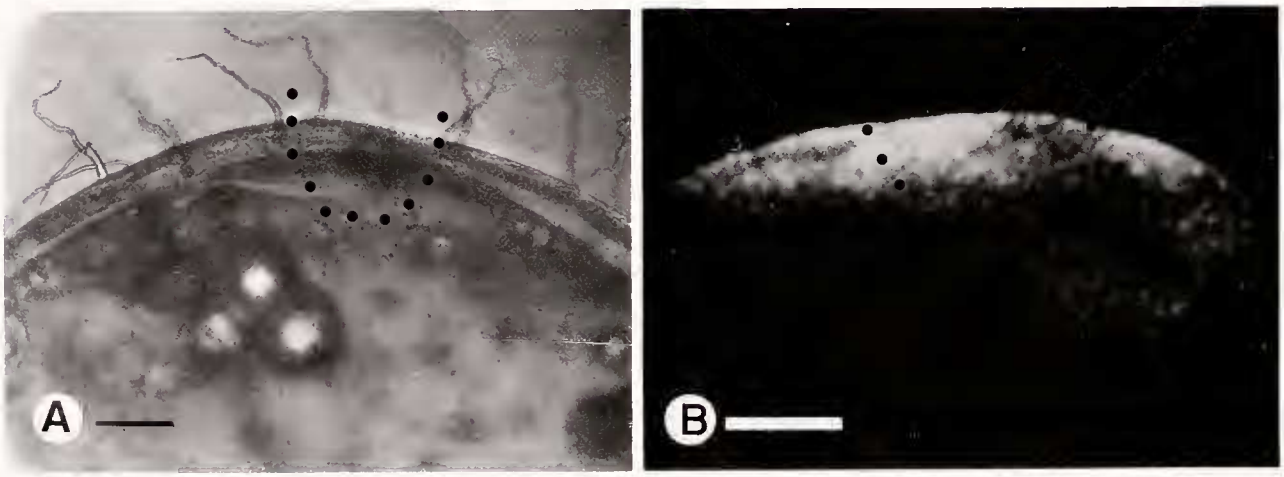

Figure 1. Accumulation of ooplasm within a UV-irradiated region of an egg. NP-EGTA/Ca^{2+} (4.2 nl) was microinjected into this egg, which was then irradiated *en profil* in a region (approximately defined by the filled circles) along a meridian and midway between its animal and vegetal poles. The UV light was projected through the 10× objective lens with no neutral density filter in the light path. Ultraviolet irradiation was intermittent, with light pulses delivered for 5 s at 2-min intervals for a total of 79 min (until T_n = 1.0). The egg was then fixed overnight at room temperature with formaldehyde dissolved in an actin-stabilizing buffer (24): 3.7% formaldehyde (Electron Microscopy Sciences, Fort Washington, PA), 100 m*M* KCl, 5 m*M* $MgCl_2$, 2 m*M* EGTA, 10 m*M* PIPES, pH 6.8. It was then dechorionated with fine forceps, permeabilized for 15 min with 0.3% Triton X-100 in BSS, and stained for 30 min in 0.25 μ*M* rhodamine phalloidin (Molecular Probes, Inc., Eugene, OR) dissolved in BSS. The irradiated region of the egg was photographed with brightfield optics just before fixation (A) and with epi-illumination after staining the egg with rhodamine phalloidin (B). Note the accumulation of ooplasm, oil droplets, and F-actin in the irradiated region. Scale bars, 100 μm.

jection site, did not undergo exocytosis. Irradiation of fertilized eggs caused ooplasm to accumulate within the irradiated region, but no such accumulations were seen in fertilized eggs that were grown in the dark; in such dark-grown eggs, the ooplasm and its contents appeared to segregate normally. Moreover, irradiation of unfertilized eggs that had not received NP-EGTA caused neither contraction of the ooplasm nor accumulation of ooplasm within the irradiated region.

All eggs that received 1.4 nl of NP-EGTA underwent cytokinesis. Of eight such fertilized eggs whose subsequent development was monitored, four hatched and the other four underwent extensive morphogenesis but did not hatch. Cleavage was abnormal in eggs that received either 4.2 or 5.6 nl of NP-EGTA, and the embryos did not develop further. Irradiation of eggs (that received 1.4 nl of NP-EGTA and were subsequently fertilized) during early cleavage caused cells in the light beam to contract globally. Moreover, irradiation of early gastrulae caused blebbing and global contraction of deep blastomeres, but irradiation of the yolk sac in stage 19, 22, and 25 embryos (that is, up to 4 days after fertilization) caused contractions that appeared similar to those seen during the rhythmic contraction waves that occur in the stellate layer of the medaka embryo (11). Cells of embryos that did not receive NP-EGTA failed to contract when they were irradiated with UV light.

Taken together, these findings show NP-EGTA to be a useful new reagent for cell and developmental biologists. Several properties of this compound—its high affinity for Ca^{2+} (6), the approximately 10,000-fold decrease in its affinity for Ca^{2+} upon photolysis (6), its weak fluorescence (6), and its persistence and low toxicity in the teleost embryo (this study)—appear to make it particularly suitable for studying events that have been linked to elevations in cytosolic $[Ca^{2+}]$: egg activation (12, 13), ooplasmic segregation (9, 10), nuclear envelope breakdown (14), mitosis (15, 16, 17), cytokinesis (8, 18), and neuronal growth cone motility (19, 20).

The accumulation of ooplasm and F-actin within the UV-irradiated region of the egg is consistent with the hypothesis that cytosolic calcium gradients organize developmental localization in eggs (10, 21, 22, 23). At present, however, the evidence consistent with this hypothesis, including that presented in the present report, is indirect and must be extended by using aequorin or a fluorescent calcium indicator to measure cytosolic $[Ca^{2+}]$ in eggs during and after photolysis of NP-EGTA. Full exploitation of the apparent promise of NP-EGTA will require the development of a dextran-conjugated form of the molecule and the exploration of wider ranges of intracellular concentrations of the cage, shapes of the irradiated region (for example, a narrow rectangle that could elevate cytosolic $[Ca^{2+}]$ in a narrow band in a cell), and light intensities.

Acknowledgments

Lionel Jaffe suggested the use of calcium cages to generate zones of elevated calcium in medaka eggs. I am grateful to Jack Kaplan and Graham Ellis-Davies for providing the NP-EGTA used in these studies; to Alan Bruns for helpful discussions about units of light intensity; and to Andrew Miller for helping to improve the text of the manuscript. This work was supported by NSF DCB-9017210 and NSF MCB-9316125.

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